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Overexpression of *Medicago sativa TMT* elevates the α-tocopherol content in *Arabidopsis* seeds, alfalfa leaves, and delays dark-induced leaf senescence

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is a major forage legume for livestock and a target for improving their dietary quality. Vitamin E is an essential vitamin that animals must obtain from their diet for proper growth and development. γ-tocopherol methyltransferase (γ-TMT), which catalyzes the conversion of δ- and γ-tocopherols (or tocotrienols) to β- and α-tocopherols (or tocotrienols), respectively, is the final enzyme involved in the vitamin E biosynthetic pathway. The overexpression of *M. sativa* L.'s γ-TMT (*MsTMT*) increased the α-tocopherol content 10–15 fold above that of wild type *Arabidopsis* seeds without altering the total content of vitamin E. Additionally, in response to osmotic stress, the biomass and the expression levels of several osmotic marker genes were significantly higher in the transgenic lines compared with wild type. Overexpression of *MsTMT* in alfalfa led to a modest, albeit significant, increase in α-tocopherol in leaves and was also responsible for a delayed leaf senescence phenotype. Additionally, the crude protein content was increased, while the acid and neutral detergent fiber contents were unchanged in these transgenic lines. Thus, increased α-tocopherol content occurred in transgenic alfalfa without compromising the nutritional qualities. The targeted metabolic engineering of vitamin E biosynthesis through *MsTMT* overexpression provides a promising approach to improve the α-tocopherol content of forage crops.

1. Introduction

Plants serve as a primary source of essential vitamins and minerals for animals. Vitamin E deficiencies in animals can lead to neurologic disorders and ataxia [1,2], impaired embryo and brain development [3,4], and even death [5]. In contrast, sufficient vitamin E intake has a number of health benefits, including reducing instances of diabetic kidney disease [6–8], and decreasing the risks of pancreatic and prostate cancer [9,10]. In addition, it can reduce animal abortion rates, improve milk quality, delay meat discoloration and extend the shelf life of meat [11–14]. Vitamin E has also played a key role in plant response to abiotic stresses. Specifically, it protects chloroplast membranes from photooxidation and provides a more optimal environment for the photosynthetic machinery by scavenging reactive oxygen species (ROS) [15,16].

Vitamin E is derived from the methylerythritol phosphate and the shikimate pathways. It is composed of four tocopherols, which possess a saturated phytyl side chain, and four tocotrienols, which have a side chain containing three desaturated carbon bonds. Depending on the number and the position of the methyl groups on the chromanol ring, the vitamin E components are classified into four classes: α-, β-, γ- and δ-tocopherols (or tocotrienols) [17]. Among these eight derivatives, α-tocopherol and α-tocotrienol have relatively higher biological activity in animals and are readily absorbed by the body [17–19]. Plants are the primary source of vitamin E, however, the content and composition of vitamin E

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varies greatly between species, tissues, and developmental stages [20–23].

There are five key enzymes involved in vitamin E biosynthesis. P-HYDROXYPHENYLPYRUVATE DIOXYGENASE (HPPD), HOMOGENTISATE PHYTYLTRANSFERASE (HPT), and TOCOPHEROL CYCLASE (TC) are the three key enzymes that regulate the total vitamin E content, while 2-METHYL-6-PHYTYLBENZOQUINONE METHYLTRANSFERASE (MPBQMT) and γ-TOCOPHEROL METHYLTRANSFERASE (γ-TMT) determine vitamin E composition. γ-*TMT* is known to alter the vitamin E composition by converting δ- and γ-tocopherol (or tocotrienol) to β- and α-tocopherol (or tocotrienol), respectively. Overexpression of γ-*TMT* leads to a complete conversion of γ-tocopherol to α-tocopherol in *Perilla frutescens*, *Arabidopsis thaliana*, *Glycine max* (soybean) and *Zea mays* (corn) [24–26]. Meanwhile, silencing γ-*TMT* via RNA interference results in a 95% reduction in the α-tocopherol levels in tobacco and increases the sensitivity to osmotic stress [27]. Additionally, the overexpression of *AtTMT* increases both salt and heavy metal tolerance in transgenic tobacco [28]. Targeted modifications of the vitamin E pathway affect both the nutritional composition and the stress tolerance, which could aid in crop improvements.

Alfalfa (*Medicago sativa* L.) is a major perennial leguminous forage crop grown worldwide. As such, it is regarded as "the queen of forage crops" because of its high biomass productivity [29]. Alfalfa provides a desirable feed for livestock due to its high content of proteins, fibers and minerals, and its favorable palatability and digestibility [30,31]. Making crop improvements in alfalfa is challenging because it is an out-crossing autotetraploid that possesses a large genome [32]. This complex genetic background has previously limited breeding efforts and the discovery and utilization of novel gene resources in alfalfa. Since the first *TMT* gene isolation, which was reported in *Synechocystis* and in *Arabidopsis* by Shintani [33], this gene has been isolated and characterized to determine compositional changes in vitamin E in favor of α-tocopherol, in many species [34–36]. Although alfalfa is a main forage crop, this approach has never been successfully attempted in alfalfa. Because of the importance of the vitamin E content in alfalfa quality and the limitations in breeding efforts, targeted approaches for engineering vitamin E biosynthesis would rapidly facilitate the availability of improved commercial alfalfa crops for animals.

In this study, we isolated and characterized a γ-TMT gene from *M. sativa* L. designated *MsTMT*. This gene is closely related to *MtTMT*, its homolog from *Medicago truncatula*. Genetic transformations using *MsTMT* to increase the expression levels of the endogenous *TMT* gene in both alfalfa and *A.thaliana* altered the vitamin E content and delayed leaf senescence in transgenic alfalfa.

2. Materials and Methods

2.1. Plant material

A. *thaliana* ecotype Columbia-0 was used for this study. Seeds were sterilized with 70% ethanol for 2 min, 5% sodium hypochlorite for 5 min, and then washed with water three times. Surface-sterilized seeds were planted on half-strength Murashige and Skoog medium (2.2 g/L Murashige and Skoog salts, pH 5.7, and 8 g/L agar), stratified at 4 °C for 3 days, and then grown under long day photoperiod conditions (16 h light/8 h dark) at 22 °C.

The alfalfa (*M. sativa* L.) used in this study was 'Zhongmu No. 1' from the Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, China. Seeds were germinated on wet filter paper and grown at 24 °C until the first true leaf emerged, at which point the seedlings were transferred to soil and grown under long day photoperiod conditions (16 h light/8 h dark) at 25 °C.

2.2. Vector construction and transformation

The γ-TMT from *M. sativa* L. was cloned based on the homologous *MtTMT* sequence from *M. truncatula* using Rapid Amplification of cDNA ends (RACE) with primers 5′-TTGTTGGTGAGTTAGCACGGGTAGC-3′ and 5′-GTTGTAGGGATTCTTCATTCGGGGC-3′. A 1,071-bp *MsTMT* opening reading frame (ORF) was amplified using primers 5′-CACGGGGGACTCTAGAATGGTGACGACAGCGAGAAT-3′ and 5′-AGGGACTGACCACCCGGGGATTGACCCTCTGCATTTTCAG-3′. The restriction enzyme recognition sites for XbaI and SmaI (underlined) were included in the ORF primers to facilitate insertion into vector pBI121 [37]. *A. thaliana* was transformed via *Agrobacterium tumefaciens* strain GV3101 by floral dip as previously described by Clough and Bent [38]. Alfalfa was transformed via *A. tumefaciens* strain EHA105 as previously described by Wright et al. [39].

2.3. Southern blotting analysis

The *MsTMT* coding sequence was labeled with digoxigenin (DIG) using a DIG nucleic acid labeling kit (Roche, Germany) and used as a hybridization probe. DNA was extracted from two-week-old *Arabidopsis* plants using a DNA Extraction Kit (Tiangen, Beijing, China). DNA (10 μg) was digested overnight with restriction enzymes EcoRI, NdeI and SpeI, after which the DNA was run on a 0.8% agarose gel, and then transferred to a Hybond-N^+ nylon membrane (Roche). The membrane was briefly washed with 2 × SSC (saline sodium citrate buffer), dried using filter paper, heated at 80 °C to fix the DNA onto the membrane and then placed into a hybridization tube with hybridization buffer and the hybridization probe. After an overnight hybridization at 42 °C, the tube was washed twice for 15 min with 2 × SSC containing 0.1% SDS (sodium dodecyl sulfate), and then twice for 15 min with 0.5 × SSC containing 0.1% SDS, before being briefly washed with washing buffer and incubated for 30 min in blocking buffer. After 30 min, the blocking buffer was poured off and an antibody solution was added for 30 min incubation. Finally, the membrane was washed twice for 15 min with washing buffer. Bands on the membrane were detected using the DIG detection kit according to the manufacturer's instructions (Roche).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated via TRIzol extraction (Life Technologies, USA), and all of the samples were treated with DNase to remove DNA contamination. Then, 1 μg total RNA was reverse transcribed into cDNA using SuperScript III (Invitrogen, USA). cDNA (5 ng) was used in a 20 μl ABI 7500 Real Time PCR System (Bio-Rad, USA) using SYBR Green PCR Master Mix (Takara, Japan). Gene-specific primers are listed in Table 1. The relative gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ method [40]. The reference genes *AT4G26410* [41] and *MsActin* were used to normalize the gene expression levels in *Arabidopsis* and alfalfa, respectively. Experiments were performed using three biological replicates and three technical replicates.

2.5. GUS histochemical assay

The GUS histochemical assay was performed according to the method described by Jefferson et al. [42]. Plant tissues were incubated at 37 °C overnight in 50 mM sodium pyrophosphate staining buffer (pH 7.2) containing 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 10 mM EDTA (ethylenediaminetetraacetic acid), 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 0.2% (v/v) Triton X-100. Additionally, 100% ethanol was used to completely remove chlorophyll from the tissues.

Table 1
Primers list.

Primers	Sequence	Application
3'RACE	TTGTTGGTGAGTTAGCACGGGTAGC	RACE
5'RACE	GTTGTAGGGATTCTTCATTCGGGGC	RACE
TMT-PBI-F	CACGGGGGACTCTAGAATGGTGACGACAGCGAGAAT	Vector construction
TMT-PBI-R	AGGGACTGACCACCCGGGGATTGACCCTCTGCATTTTCAG	Vector construction
35S-PBI-F	GGTGGCTCCTACAAATGCCA	Genotyping
GUS-PBI-R	GAAACGCAGCACGATACGC	Genotyping
TMT-GFP-F	CATGCCATGGATGGTGACGACAGCGAGAAT	Vector construction
TMT-GFP-R	GGACTAGTTTGACCCTCTGCATTTTCAGGC	Vector construction
AtHPPD-F	GCGGTTTAAATTCAGCGGTCCTG	Q-PCR
AtHPPD-R	TCCGTGCACTGGCTCGTTAATC	Q-PCR
AtHPT-F	TGGAGCAAAGTCATCTCGGTTG	Q-PCR
AtHPT-R	GACTTAGCTCGAGCCCACAAAG	Q-PCR
AtTC-F	TGTCCGAAGGGTTCCAAGCTAC	Q-PCR
AtTC-R	CACAGTTTCCGCATAGTCAGTACG	Q-PCR
AtMPBQMT-F	TGCCGGTTTCAAGGACGTTCAG	Q-PCR
AtMPBQMT-R	TCTTCCTTTGGACCAAGCTGGAG	Q-PCR
AtTMT-F	TGCTCAATCACTCGCTCATAAGGC	Q-PCR
AtTMT-R	TCTTCGAATGGCTGATCCAACGC	Q-PCR
Reference-F	GAGCTGAAGTGGCTTCCATGAC	Q-PCR
Reference-R	GGTCCGACATACCCATGATCC	Q-PCR
MsActin-F	GAGCGTTTCCGTTGTCCTGA	Q-PCR
MsActin-R	AGGTGCTGAGGGAAGCCAAA	Q-PCR
MsTMT-F	ATGTTGGGTGTGGCATAGGG	Q-PCR
MsTMT-R	AGGGCTGAGAGTGATGCCTA	Q-PCR
DREB2A-F	TCGAGCTGAAACGGAGGTAT	Q-PCR
DREB2A-R	GACCTAAATGGCGACGATGT	Q-PCR
LEA7-F	GTGAGCAGGTGAAACAGATGGC	Q-PCR
LEA7-R	AGTGCGAAGCCCTAAAGTGTGC	Q-PCR
RAB18-F	ACATGGCGTCTTACCAGAACCG	Q-PCR
RAB18-R	TACTGCTGCTGGATCGGGTTTC	Q-PCR
TMT gsp1	CTGAGCAGCACGATGATCAGAAACAGA	Genome walking
TMT gsp2	TAGGTACACACGAAACTACGGTGCGA	Genome walking
TMT gsp3	CTTACACATTAATAGAAGGCAAGCGGAC	Genome walking

2.6. Extraction of tocopherols, tocotrienols and the HPLC analysis

Analyses of tocopherol and tocotrienol levels were performed according to a modified procedure described by Zhang et al. [43]. Briefly, for seed tissues, seeds were dried at room temperature for two weeks after being harvested. Then, 10 mg of seeds or 50 mg of fresh leaves were ground in liquid nitrogen and homogenized in 1 ml methanol: chloroform (2:1, v/v) extraction buffer containing 0.01% butylated hydroxytoluene. The homogenized tissue was incubated for 20 min at room temperature. Then, 300 μl chloroform was added, followed by 600 μl H_2O. The samples were mixed well before being centrifuged at 14,000 × *g* for 10 min. The upper aqueous phase was discarded and the bottom organic phase was transferred to a new tube and then dried in a vacuum. The dried samples were resuspended in 400 μl dichloromethane: methanol (1:5, v/v) or 400 μl n-heptane for the tocopherol and tocotrienol HPLC analysis, respectively. For tocopherol analysis, 20 μl of the sample was injected into a Shiseido-C18 column (4.6 × 150 mm length, 5 μm particle size) using methanol:water (97:3, v/v) as the mobile phase with a flow rate of 1.2 ml/min. Sample components were detected and quantified by fluorescence with excitation at 292 nm and emission at 330 nm. For tocotrienol analysis, samples were injected into an inertsil-silica column (4.6 × 250 mm length, 5 μm particle size) using n-heptane:isopropanol (99:1) as the mobile phase and a flow rate of 1 ml/min. Tocopherols and tocotrienols were identified by retention times and were quantified relative to an external individual vitamin E compound standard.

2.7. Biomass measurements and analyses of the expression levels of several osmotic marker genes

Seeds from transgenic and control lines were harvested at the same time and dried at room temperature for two weeks before being stratified at 4 °C for 3 days and then germinated on half-strength Murashige and Skoog medium. After two weeks, seedlings were grown on half-strength MS medium with or without 100 mM mannitol for 7 days, at which point the biomass was measured. Each biomass measurement was performed on four biological replicates with pools of 12 seedlings for each sample. qRT-PCR was used to quantify expression levels of genes responsive to osmotic stress.

2.8. Dark-induced alfalfa leaf senescence

Alfalfa leaves were detached from 2-month-old transgenic and control plants. These leaves were then surface-sterilized with 70% ethanol for 2 min and mercuric chloride for 5 min, before being washed three times with water and placed on half-strength MS medium. These plates were then wrapped in aluminum foil and incubated at 24 °C for 4 d.

2.9. Fiber and crude protein analyses

Fiber and crude protein analyses were performed according to the method described by Bruno et al. [44].

2.10. Statistical analysis

All of the experiments were performed with at least three biological replicates. Data were expressed as mean ± standard deviation (SD). These analyses were carried out via a two-tailed Student's *t* tests with a significance of $P < 0.05$.

3. Results

3.1. Cloning and sequence analysis of MsTMT from M. sativa L.

γ-TMT, which catalyzes the conversion of δ- and γ-tocopherols (or tocotrienols) to β- and α-tocopherols (or tocotrienols), respec-

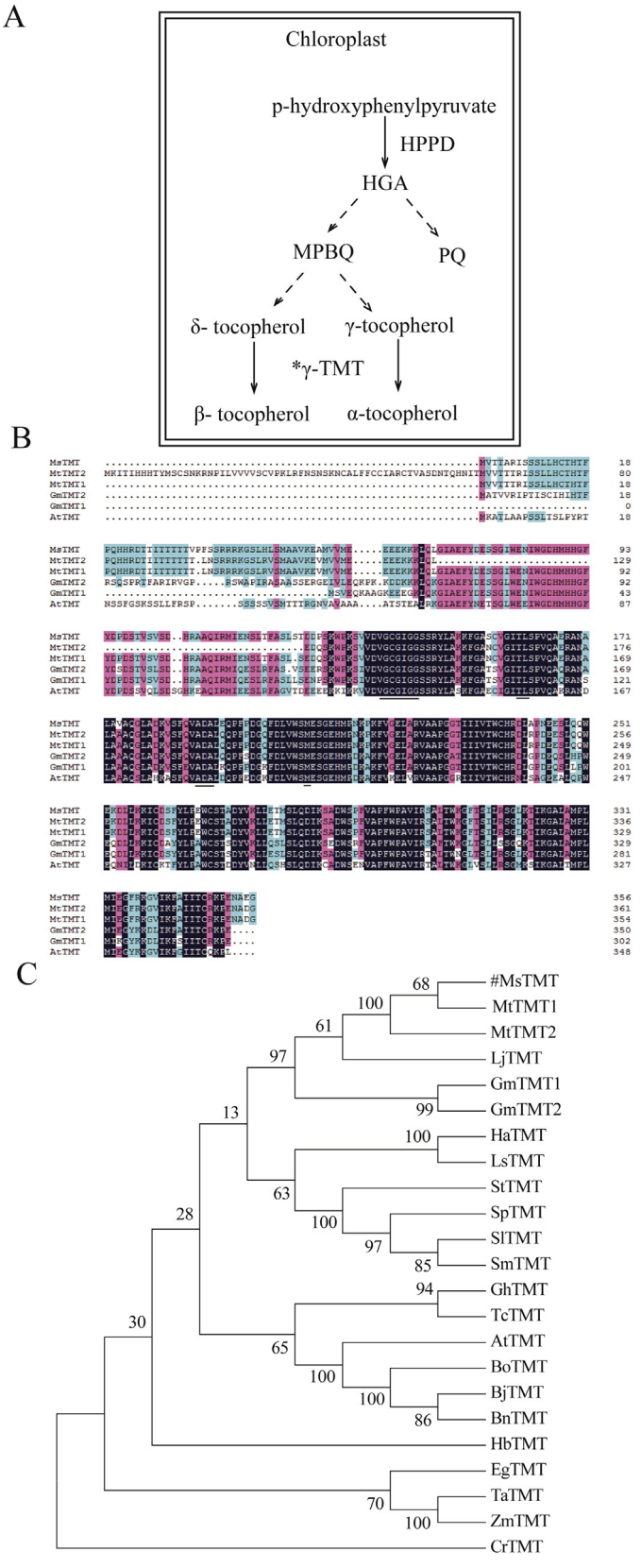


Fig. 1. A. Simplified tocopherol biosynthetic pathway in higher plants.
HPPD: P-hydroxyphenylpyruvate dioxygenase; HGA: homogentisic acid; MPBQ: 2-methyl-6-phytyl-1, 4-benzoquinone; γ-TMT: γ-tocopherol methyltransferase; PQ: plastoquinone. The target gene is marked with *.
B. Multiple sequence alignment of TMT from different species.
AtTMT ACO57634.1 [*Arabidopsis thaliana*]; MtTMT2 AAX63740.1 [*Medicago truncatula*]; MtTMT1 KEH39887.1 [*Medicago truncatula*]; GmTMT2 AAX63899.1 [*Glycine max*]; GmTMT1 BAK57290.1 [*Glycine max*]; MsTMT [*Medicago sativa*].

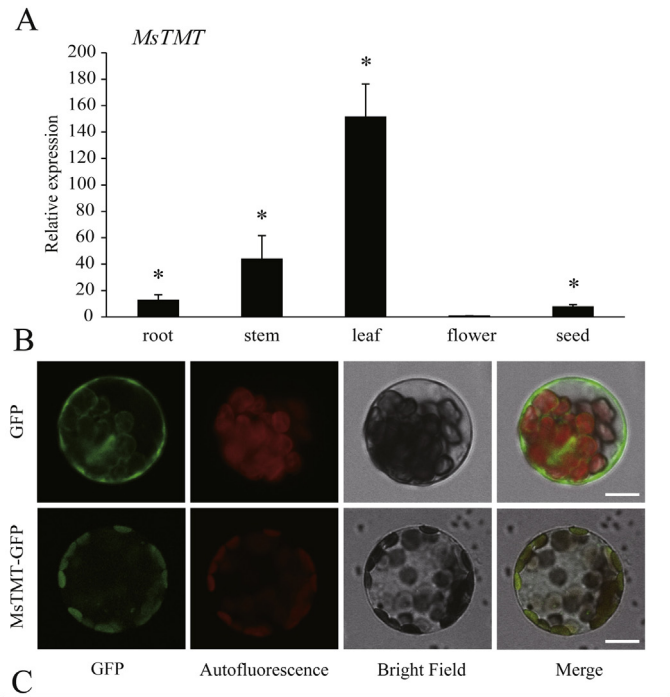
tively, was chosen as the target gene (Fig. 1A). TMT sequences downloaded from NCBI were aligned, and accordingly, a conserved fragment was selected to perform RACE. The full-length *MsTMT* sequence was obtained by combining 660-bp and 743-bp fragments obtained via 3′ and 5′ RACE, respectively. The results from several repeated RACE approaches established the presence of a single *TMT* gene in alfalfa leaves. The aforementioned full-length *MsTMT* fragment contained a 1,071-bp ORF encoding a protein of 356 amino acids in length that belongs to the AdoMet-dependent methyltransferase superfamily and has four S-adenosylmethionine binding sites. A multiple sequence alignment revealed that MsTMT shares high sequence similarities with TMTs from other species, including the four conserved S-adenosylmethionine binding sites (Fig. 1B). A phylogenetic tree analysis indicated that MsTMT is closely related to MtTMT from *M. truncatula*, with identities of 93.84% and 64.85% to MtTMT1 and MtTMT2, respectively (Fig. 1C).

3.1. *Expression pattern and subcellular localization of* MsTMT

To determine the *MsTMT* expression pattern, quantitative real-time PCR was carried out in the root, stem, leaf, flower and seed. While *MsTMT* is constitutively expressed in all of the tissues examined, the transcript levels were most abundant in the leaves and least abundant in the flowers. Specifically, the *MsTMT* expression levels were ~150 fold higher in leaves compared to the corresponding expression levels in the flowers (Fig. 2A). Furthermore, transient expression of *MsTMT* was performed in *Arabidopsis* protoplasts. The empty vector control exhibited GFP signal throughout the protoplasts. However, GFP signal was detected only in the chloroplasts with vector harboring MsTMT protein (Fig. 2B).

To better understand the molecular components regulating *MsTMT* gene expression, a 1.5 kb fragment of the gene's native promoter was cloned with the appropriate primers (Table 1). Subsequent analysis using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [45], identified 27 putative regulatory *cis*-elements in the promoter, including 18, 6 and 3 *cis*-elements related to light-responsiveness, hormone-responsiveness and circadian control (Fig. 2C). Due to the presence of a number of light-responsive *cis*-elements in the *MsTMT* promoter, *MsTMT* expression levels in alfalfa were examined in tissues grown for 48 and 72 h in continuous dark and subsequently exposed to light for 0.25, 0.5, 6, 12, 24, 48, 72 h. The data clearly showed that *MsTMT* expression levels increased significantly in response to continuous darkness. However, upon light exposure, the expression levels of *MsTMT* dropped sharply at 0.25 h, and continued to decrease with the increasing time of light treatment. Specifically, *MsTMT* expression decreased significantly at 12 and 24 h and then recovered to control level at 48 and 72 h (Fig. 2D). These data sug-

S-adenosylmethionine binding sites are underlined. Colors highlighted homology levels. Black represents identity = 100%, red represents identity > = 75%, green represents identity > = 50%.
C. Phylogenetic tree analysis of MsTMT and other TMT proteins.
Neighbor-joining method was used to construct the tree using protein sequences of TMT downloaded from NCBI as follows:
AtTMT ACO57634.1 [*Arabidopsis thaliana*]; BjTMT ABI23433.1 [*Brassica juncea*]; BnTMT ABF56215.1 [*Brassica napus*]; BoTMT AAO13806.1 [*Brassica oleracea*]; EgTMT AEU17779.1 [*Elaeis guineensis*]; GmTMT1 BAK57290.1 [*Glycine max*]; GmTMT2 AAX63899.1 [*Glycine max*]; GhTMT ABE41798.1 [*Gossypium hirsutum*]; HaTMT AEX55701.1 [*Helianthus annuus*]; HbTMT BAH10645.1 [*Hevea brasiliensis*]; LsTMT ADC91915.1 [*Lactuca sativa*]; LjTMT AAY52459.1 [*Lotus japonicus*]; MtTMT1 KEH39887.1 [*Medicago truncatula*]; MtTMT2 AAX63740.1 [*Medicago truncatula*]; SlTMT AEO80033.1 [*Solanum lycopersicum*]; SmTMT ADD64697.1 [*Solanum melongena*]; SpTMT ADZ24710.1 [*Solanum pennellii*]; StTMT ADV36922.1 [*Solanum tuberosum*]; TcTMT XP_007029706.1 [*Theobroma cacao*]; TaTMT CAI77219.2 [*Triticum aestivum*]; ZmTMT AGF92812.1 [*Zea mays* subsp. mays]; CrTMT CAI59122.1 [*Chlamydomonas reinhardtii*]. Bootstrap test (500 replicates) is shown on the branches.



C

Site name	Sequence	Function	No.
ABRE	TACGTG	Involved in the abscisic acid responsiveness	1
TGA-element	AACGAC	Auxin-responsive element	1
circadian	CAANNNNATC	Involved in circadian control	3
Box 4	ATTAAT	Involved in light responsiveness	2
Box 1	TTTCAAA	Involved in light responsiveness	4
ACE	AAAACGTTTA	Involved in light responsiveness	1
G-Box	CACGTA	Involved in light responsiveness	3
GT1-motif	GGTTAA	Involved in light responsiveness	5
Gap-box	CAAATGAA(A/G)A	Involved in light responsiveness	1
MNF1	GTGCCC(A/T)(A/T)	Involved in light responsiveness	1
as-2-box	GATAATGATG	Involved in light responsiveness	1
CGTCA-motif	CGTCA	Involved in the MeJA-responsiveness	2
TGACG-motif	TGACG	Involved in the MeJA-responsiveness	2

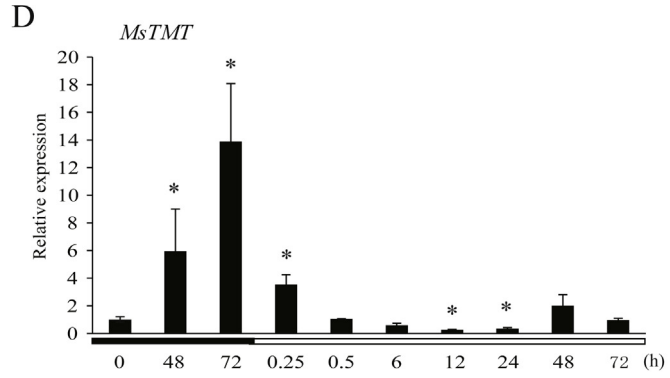


Fig. 2. A. Tissue specific expression pattern of *MsTMT* in alfalfa.
Samples of different organs were collected from 2-year-old alfalfa plants. Data are mean ± SD of three biological replicates with three technical replicates.
B. Subcellular localization of MsTMT protein.
Transient expression of MsTMT-GFP and GFP alone in Arabidopsis protoplasts. GFP: Green Fluorescent Protein. Bar: 10 μm.
C. Cis-elements analysis of *MsTMT* promoter.
D. Expression levels of *MsTMT* in response to light treatment.
2-week-old alfalfa seedlings were exposed to dark treatment, and then transferred to light condition. Samples were collected at different time points. The rest were performed the same as Fig. 2 A. Black bar represents dark treatment, white bar represents light treatment.

gest that light negatively regulates the expression of *MsTMT* and this negative regulation is reversible.

3.2. MsTMT *transcript and protein levels in transgenic* Arabidopsis

Vector pBI121 was used to express *MsTMT*, which was followed by the GUS reporter gene, under the transcriptional control of the constitutive CaMV 35S promoter. This 35S:*MsTMT* construct and the pBI121 empty vector were both transformed into *Arabidopsis*. A PCR analysis was performed to identify homozygous transgenic lines using the listed primers (Table 1). In total, seven independent transgenic lines were confirmed as containing *MsTMT* (Fig. 3A). No visible phenotypic differences were observed between the transgenic plants harboring *MsTMT* and those containing the control pBI121 empty vector under standard culture condition (data not shown). The integration of *MsTMT* was confirmed by southern blot analysis of genomic DNA using a probe specific against *MsTMT* without overlapping with the corresponding endogenous gene in all of the transgenic lines. Amongst all of the tested lines, lines 1, 3, 7 and 8 (designated OE1, OE3, OE7 and OE8, respectively) were single copy. We specifically selected to continue with OE1, OE3 and OE8 lines, since the OE7 plants didn't produce enough seeds, although they were all grown under the same conditions (Fig. 3B). In the transgenic lines, fragments of different sizes were observed because of the use of different restriction endonuclease and the inevitable random insertion sites of *MsTMT* into the *Arabidopsis* genome. Quantitative real-time PCR was performed to examine the transcript levels of the introduced *MsTMT* in *Arabidopsis,* confirming that *MsTMT* was highly expressed among the transgenic lines (Fig. 3C). Approximate protein expression levels were determined based on the aforementioned GUS translational fusion. The data showed higher GUS expression in primary roots, lateral roots, cotyledons and true leaves in transgenic lines as compared to the corresponding pBI121 empty vector control plants (Fig. 3D). Moreover, GUS expression was expressed highly in transgenic *Arabidopsis* seeds as compared to control seeds (Fig. 3E). Overall, OE1 and OE8 had higher GUS expression levels than OE3, which clearly illustrated the concordance of MsTMT protein levels with its transcript levels.

3.3. *Expression levels of endogenous genes involved in vitamin E biosynthesis in transgenic* Arabidopsis.

To explore the potential negative/positive feedback caused by overexpressing *MsTMT* on the expression levels of endogenous vitamin E biosynthetic pathway genes, we examined the expression levels of principal endogenous vitamin E biosynthesis-related genes in transgenic *Arabidopsis*. The expression levels of all the tested genes were not significantly altered in the transgenic lines relative to the corresponding control plants (Fig. 4).

3.4. *Detection of the tocopherol and tocotrienol contents in transgenic* Arabidopsis *seeds and leaves.*

Previous GUS staining results have showed that MsTMT protein was highly expressed in transgenic *Arabidopsis* seeds and leaves (Fig. 3D, E). To further explore the function of *MsTMT,* we examined tocopherol and tocotrienol levels in transgenic and wild type *Arabidopsis* seeds and leaves. In *Arabidopsis* seeds, the overexpression of *MsTMT* converted a majority of the γ-tocopherol to α-tocopherol. As a result, the transgenic lines contained at least 10 times more α-tocopherol than the corresponding wild type plants (Fig. 5A). Interestingly, the α-tocotrienol content was not altered, while the β-tocotrienol level was significantly increased in transgenic lines compared with wild type plants. This change in the vitamin E com-

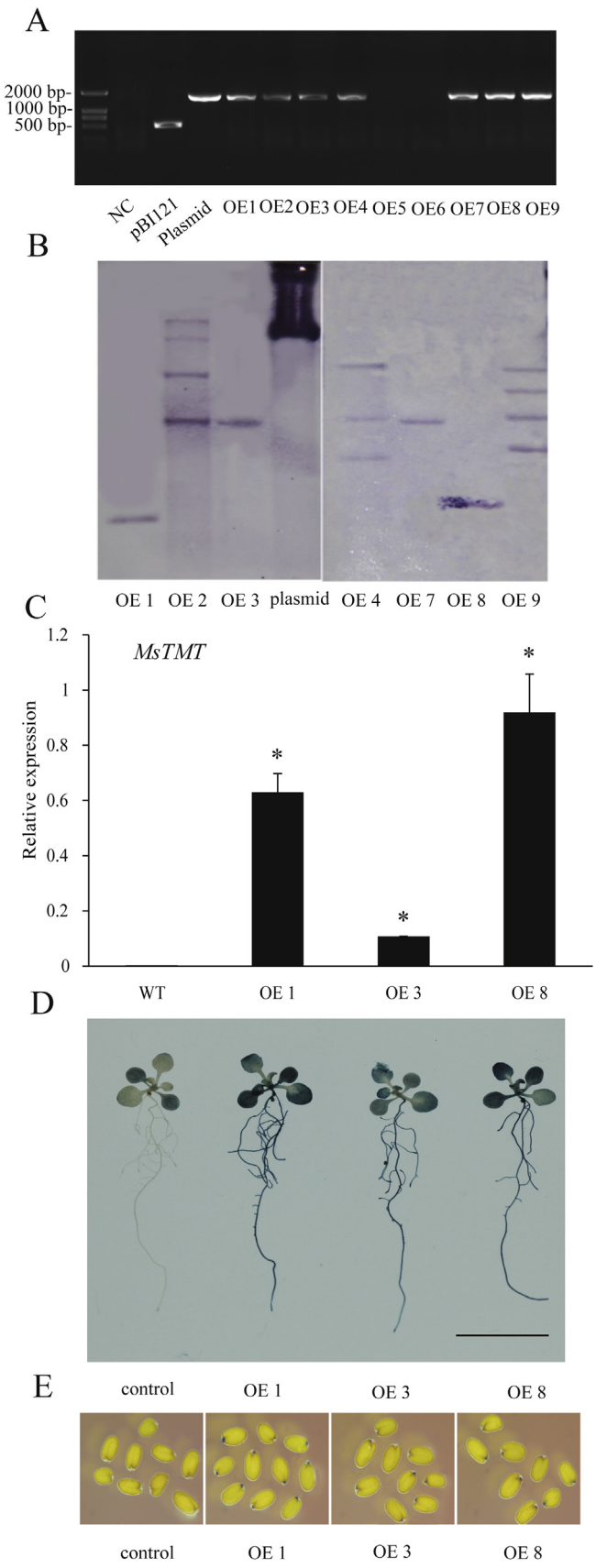


Fig. 3. A. Molecular confirmation of transgenic *Arabidopsis* lines.
DNA was extracted from 2-week-old *Arabidopsis* seedlings for PCR analysis. NC: Negative control; pBI121: Empty vector; Plasmid: Positive control.
B. Southern blot analysis of *MsTMT* overexpression *Arabidopsis* (OE1-4, 7-9). DNA was extracted from 2-week-old *Arabidopsis* seedlings. Plasmid was the positive control. OE 1, Plasmid and OE8 were digested by EcoR I; OE 2, OE 4 and OE 9 were digested by Nde I; OE 3 and OE 7 were digested by Spe I. Coding sequence of *MsTMT* was used as hybridization probe.
C. Expression analysis of *MsTMT* in *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type control plants by qRT-PCR.
Total RNA was extracted from 2-week-old *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and control seedlings and then subjected to qRT-PCR analysis. The expression levels of *MsTMT* were normalized to At4g26410. Data are mean ± SD of three biological replicates with three technical replicates.
D. Histochemical GUS staining of 10-d-old *MsTMT* overexpression *Arabidopsis* seedlings (OE 1, OE 3 and OE 8) and wild type seedlings with pBI121 empty vector in Col-0 background. Bar: 1 cm.
E. Histochemical GUS staining in dry seeds of MsTMT overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type with pBI121 empty vector in the Col-0 background.

position had no effect on the total vitamin E content in transgenic plants (Fig. 5B), suggesting the rechanneling of substrate from γ-tocopherol to α-tocopherol rather than altering the total vitamin E content occurs. Similarly, in the leaves of transgenic *Arabidopsis*, the global level of β- + γ-tocopherols was significantly decreased when compared with wild type plants. Since there was no β-tocopherol accumulated in *Arabidopsis* leaves as reported [17], γ-tocopherol must be decreased (Fig. 5C). δ-, γ- and β-tocotrienols were undetectable, and the α-tocotrienol and total vitamin E contents were not affected (Fig. 5D). Thus, *MsTMT* efficiently altered the flux in favor of α-tocopherol in transgenic *Arabidopsis* without altering the total vitamin E content.

3.5. *Evaluation of the osmotic stress tolerance of transgenic* Arabidopsis.

Vitamin E is known to play a role in plant stress responses [46], therefore, we evaluated the osmotic stress tolerance of transgenic and wild type *Arabidopsis* by measuring plant biomass and the expression levels of osmotic stress responsive marker genes. As shown in Fig. 6A, transgenic and wild type lines displayed similar biomass in the absence of mannitol, however, when osmotically stressed, transgenic lines exhibited higher biomass as compared to the wild type lines. Molecular analyses focused on the expression levels of established osmotic stress responsive genes such as *DREB2A*, *GRF7*, *AREB1*, *LEA7*, *ABF3*, *RD29A*, *RD22*, *ABI5*, *RD20*, *RD29B* and *RAB18* were performed [47–49]. Amongst these genes, a significant increase in the transcript levels of *DREB2A*, *LEA7* and *RAB18* were observed in all the transgenic lines (Fig. 6B).

3.6. *Overexpression of* MsTMT *increased the contents of α-tocopherol and α-tocotrienol and delayed leaf senescence in transgenic alfalfa.*

To further explore the role *MsTMT* potentially plays in improving alfalfa quality, a 35S:MsTMT vector was constructed and introduced into alfalfa plants (*M. sativa* L. cv. Zhongmu No1.) via *A. tumefaciens*-mediated transformation. Eleven independent lines were verified to harbor the construct using primers 35S-PBI-F and GUS-PBI-R (Fig. 7A). Quantitative real-time PCR analysis revealed that transgenic lines OE5 and OE16 had significantly higher transcript levels of *MsTMT* (Fig. 7B). GUS histochemical staining confirmed the increased protein levels in these lines, and the older leaves were found to have higher GUS activities when compared with new leaves. Generally speaking, the GUS activity levels in all of the transgenic plants were higher than in wild type plants (Fig. 7C). There was no visible morphological differences between transgenic and wild type alfalfa plants under standard culture condition (data not shown). Tocopherols and tocotrienols were analyzed using leaves cut from the same part of the stem. A modest, but significant, increase in α-tocopherol was observed in transgenic lines OE5 and OE16 relative to wild type plants, implying that γ-tocopherol was converted to α-tocopherol (Fig. 7D), which was in line with both the transcriptional levels and GUS staining results. All of the tocotrienols were detected in alfalfa (Fig. 7E, S1).

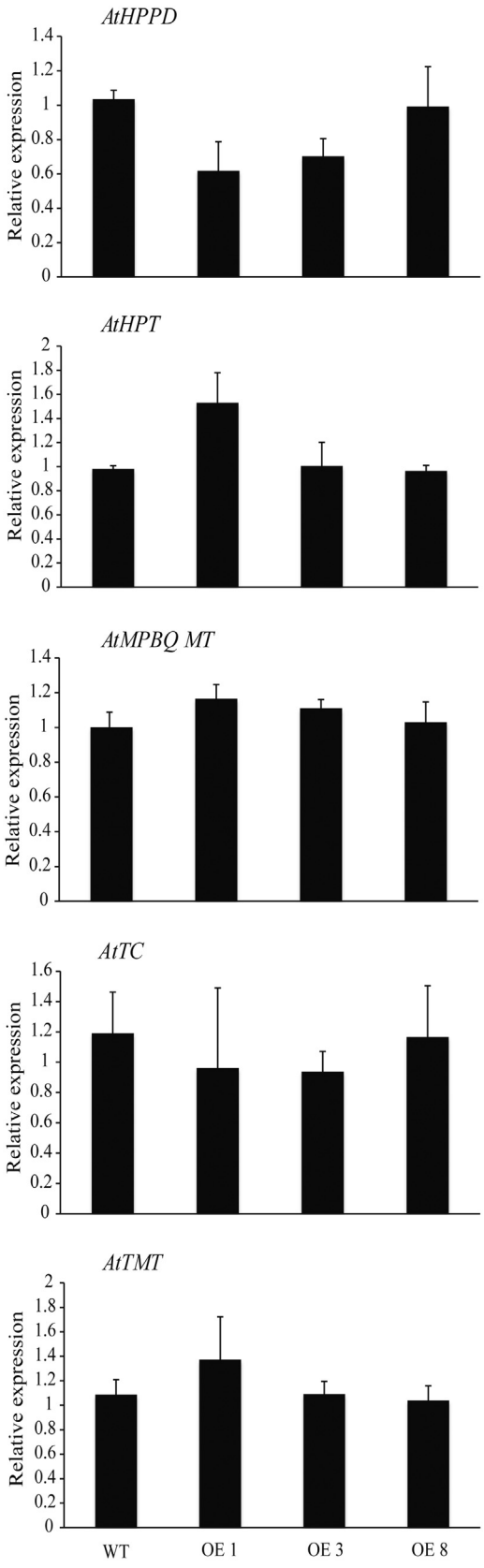


Fig. 4. Expression analysis of endogenous genes involved in vitamin E biosynthesis in *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type control. 2-week-old *Arabidopsis* seedlings were used for the qRT-PCR. Data are mean ± SD with three biological replicates and three technical replicates. The expression levels of the genes detected in the experiment were normalized to At4g26410.

Surprisingly, the α-tocotrienol content was increased 0.6–2.4 fold in transgenic lines compared with wild type plants (Fig. 7E); however, no significant differences were observed in other tocotrienols. The total vitamin E content was calculated by taking the sum of all eight compounds and no significant differences were found in the total vitamin E content (Fig. S1).

Vitamin E is involved in leaf senescence [50], and our prior results showed increased *MsTMT* expression levels in response to darkness (Fig. 2D). To explore this phenomenon further, we performed a dark-induced senescence study to determine the possible correlation between the elevated *MsTMT* transcript level and the subsequently elevated α-tocopherol and α-tocotrienol contents during alfalfa leaf senescence. Within the transgenic alfalfa, GUS activity was comparatively low in the younger leaves compared with older leaves, therefore, the older leaves were employed in these studies. Interestingly, the initiation of senescence occurred from the center to the edge of the leaves. After 4 days in the dark, wild type leaves were completely chlorotic, however, this symptom was delayed in the transgenic lines (Fig. 7F).

3.7. Nutritional qualities of transgenic alfalfa were not compromised

To evaluate the potential application of *MsTMT* overexpression in alfalfa, the nutritional qualities of the transgenic lines were measured. Nutritional quality analyses revealed that the transgenic lines had increased crude protein (CP) content, ranging from 17.7% to 24.1%, in OE5 and OE10 compared with the control line. Other nutritional quality traits, including the acid and neutral detergent fibers (ADF and NDF, respectively), did not show differences between the transgenic lines and the control plants (Fig. 8).

4. Discussion

Vitamin E is a necessary dietary factor for animals, and among its compounds, α-tocopherol was shown to be the only one that meets animal vitamin E requirements [5]. In this study, we isolated an open reading frame of 1,071 bp in length from alfalfa, encoding γ-TMT, which catalyzes the last step of vitamin E biosynthesis by converting γ- and δ-tocopherols (or tocotrienols) to α- and β-tocopherols (tocotrienols), respectively. The amino acid sequence alignment and phylogenetic tree analyses suggested that γ-TMT was conserved among species, and it was closely related to MtTMT from *M. truncatula* and GmTMT from *G. max*. α-tocopherol is the predominant form of vitamin E in leaves [17], aligned with the *MsTMT* expression analysis data from leaf tissue and the chloroplasts localization (Fig. 2A,B). This is the first report on the isolation of the gene involved in vitamin E biosynthesis from the forage legume alfalfa.

To explore the function of *MsTMT*, it was overexpressed in *Arabidopsis*. In the resulting transgenic *Arabidopsis* seeds, the α-tocopherol level was found to have increased significantly. The ratio of α-tocopherol to total vitamin E increased from 1.5% to 37%, indicating that γ-TMT activity was limited in wild type *Arabidopsis* seeds, and that it was not capable of converting all of the γ-tocopherol to α-tocopherol. Thus, more γ-tocopherol accumulated in seeds. Similar results have been observed in *Latuca sativa* L, *Brassica juncea*, *Oryza sativa* (rice), and *G. max* (soybean) [34–36,51]. Zhang et al. [36] reported that the overexpression of *AtTMT* in rice increased the α-tocotrienol content in seeds; however, in the present study, β-tocotrienol was increased in the transgenic lines rather than α-tocotrienol. This result suggested that TMT proteins from different organisms might regulate specific preferences with regards to the composition of tocopherols or tocotrienols.

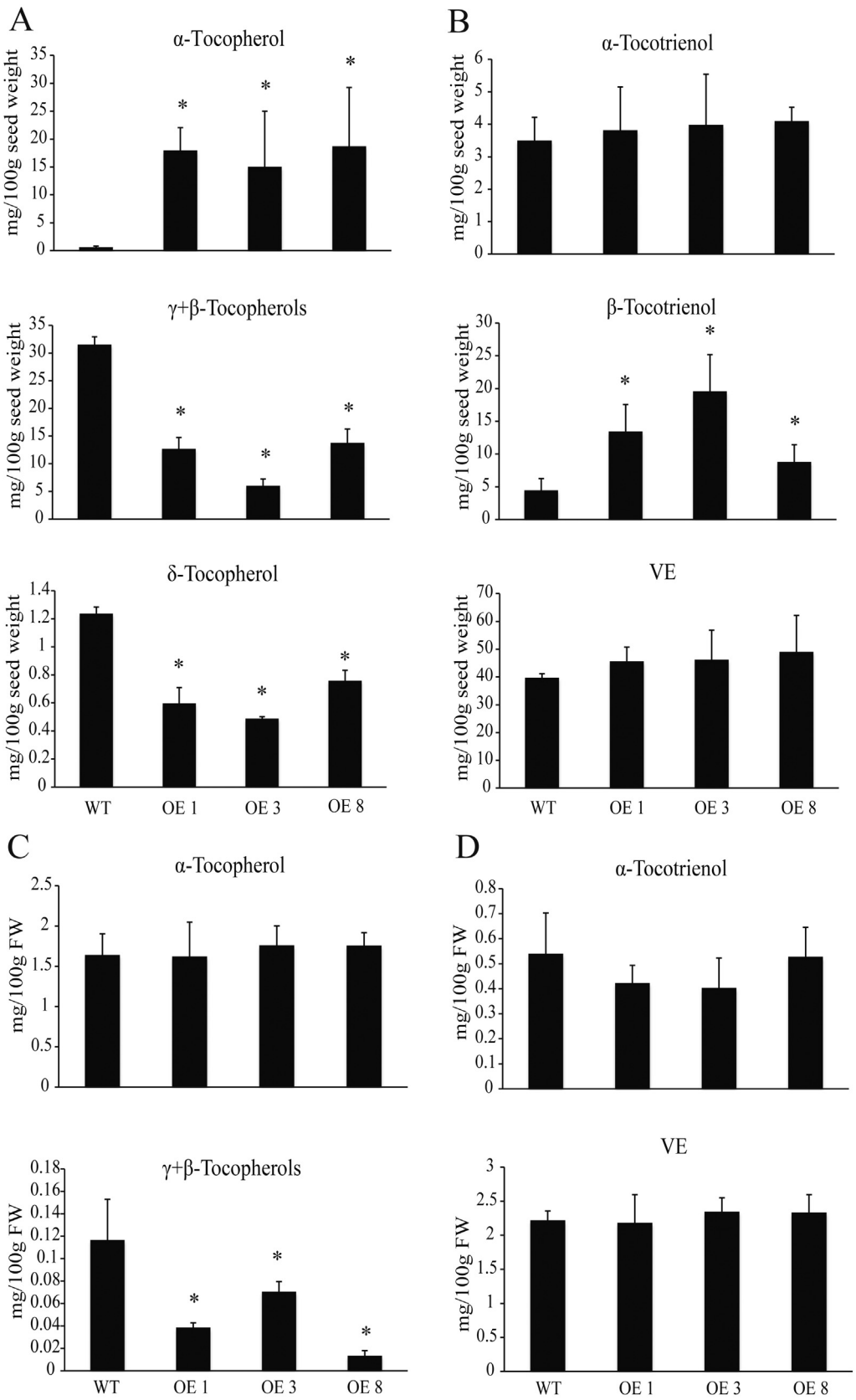


Fig. 5. Tocopherols and tocotrienols levels of *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type in seeds and leaves. 2-week-old plants and fully matured seeds were harvested and used in the analyses. Results presented as mean ± SD. Each with four biological replicates. VE: Vitamin E.
A. Tocopherols B. Tocotrienols and total vitamin E levels in *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type seeds.
C. Tocopherols D. Tocotrienols and total vitamin E levels in *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type leaves.

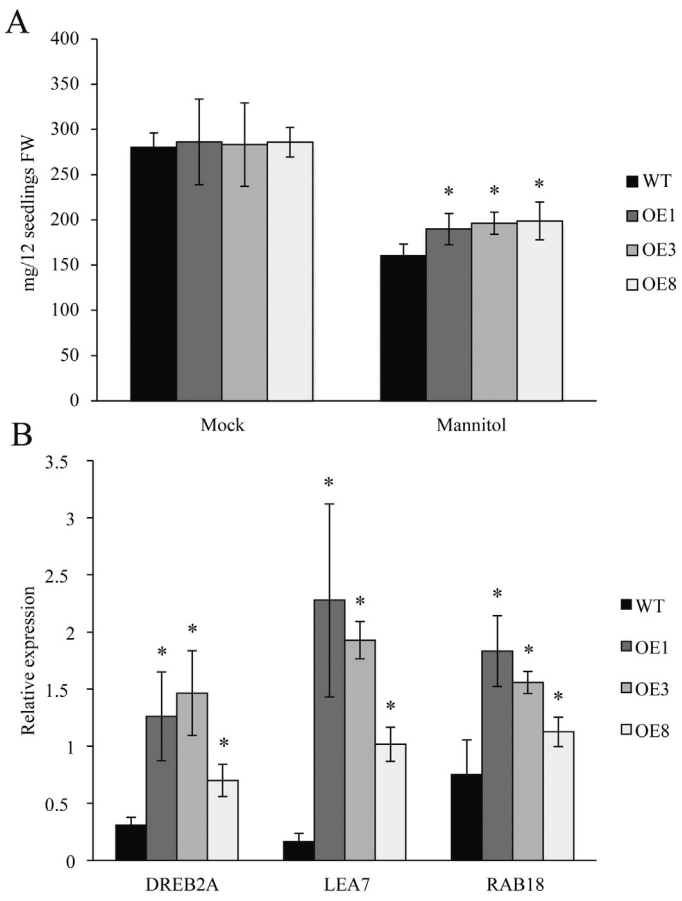


Fig. 6. A. Biomass of *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type control with or without mannitol treatment.
2-week-old *Arabidopsis* seedlings were treated with or without 100 mM mannitol for 7 d and harvested. 12 seedlings were used as one replicate, each was performed with four biological replicates. Data are mean ± SD.
B. Expression levels of osmotic marker genes in *MsTMT* overexpression *Arabidopsis* (OE 1, OE 3 and OE 8) and wild type control with mannitol treatment. Data are mean ± SD, each was performed with three biological replicates. 2-week-old *Arabidopsis* seedlings were treated with 100 mM mannitol for 7 d and used for expression analysis.

In our study of *Arabidopsis* leaves, γ-tocopherol was decreased significantly, however, the α-tocopherol content did not change in the transgenic lines, which was consistent with the results observed by Jin and Daniell [28]. However, Zhang et al. [24] showed an increased α-tocopherol content in transgenic *Arabidopsis* leaves via overexpression of *GmTMT2a* from soybean. This discrepancy may due to different TMT activities in different species and subtle differences in their specific roles with regard to controlling vitamin E biosynthesis. Regardless, α-tocopherol accounted for up to 90% of tocopherols in wild type *Arabidopsis* leaves [17], and even though γ-tocopherol was completely converted to α-tocopherol, it was still difficult to obtain a substantial amount of α-tocopherol. *MsTMT* expression and subsequent protein levels were highly increased in transgenic lines, however, the endogenous vitamin E biosynthetic genes *AtMPBQMT* and *AtTMT*, which are considered to be responsible for vitamin E composition, were not altered. This indicates that the changes in vitamin E composition observed in this study were due to *MsTMT* overexpression, and not due to changes in these endogenous genes. Furthermore, the total vitamin E content was not changed in transgenic *Arabidopsis,* which correlated with the unaltered *AtHPPD*, *AtHPT* and *AtTC* expression levels, which determine vitamin E content. Overall, *MsTMT* is responsible for vitamin E composition, not the content. Heterologous overexpression of *MsTMT* did not affect these endogenous gene expressions.

Due to the α-tocopherol level being significantly higher in the seeds of all transgenic *Arabidopsis* lines, we examined the biomass and expression levels of osmotic stress responsive marker genes for transgenic and wild type *Arabidopsis* under mannitol treatment. The biomass was significantly higher in transgenic lines when compared with the control under mannitol treatment, suggesting that α-tocopherol plays a role in plants response to osmotic stress. In addition, the expression levels of osmotic marker genes *DREB2A*, *LEA7* and *RAB18* were all significantly increased in transgenic lines compared to wild type. It is established that *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A* (*DREB2A*) is an important osmotic stress responsive gene, and overexpression of *DREB2A* leads to an induction of a cascade of downstream genes and by extension significant drought stress tolerance [48,49]. *RESPONSIVE TO ABA 18* (*RAB18*) is a representative ABA-inducible gene [52], and an enhanced *RAB18* expression level suggests the involvement of ABA signaling pathway in response to osmotic stress in the transgenic lines. In addition, *LATE EMBRYOGENESIS ABUNDANT 7* (*LEA7*), like all *LEA* proteins in plants, is associated with tolerance against stresses, and *LEA7* being known to stabilize enzymes during drying conditions [53]. A 10 fold increase of *LEA7* expression in our transgenic lines in response to mannitol treatment corroborates the report, and suggests a role of *LEA7* gene product in enhancing resistant in the transgenic lines. Taken together, our data showed that α-tocopherol potentially improves the osmotic stress tolerance of transgenic *Arabidopsis* by regulating the expression levels of osmotic stress marker genes as well as an increase in biomass.

Since *MsTMT* was functional in *Arabidopsis*, we overexpressed it in alfalfa. As expected, increased α-tocopherol level was observed in lines 5 and 16, which is in alignment with the transcript levels and GUS staining results of *MsTMT* in the transgenic lines. Intriguingly, we observed an increased α-tocotrienol level in line 10, which did not significantly overexpressed *MsTMT*. This discrepancy may due to the different posttranscriptional modification of *MsTMT* in different transgenic lines, which could lead to different enzyme activity, thus resulting in alteration of vitamin E composition. Similarly, Zhang et al. [24] overexpressed *GmTMT2a* from soybean (*Glycine max*) in *Arabidopsis* and observed that the transcriptional levels among transgenic lines were similar while a significant difference of tocopherol content in these transgenic lines occurred. Likewise, when overexpressed *AtTMT* in rice, similar tocopherol levels were observed in all the transgenic lines while a huge expression variation among the transgenic lines occurred [54]. This increase in the level of tocotrienol is intriguing, since tocotrienols generally accumulate in seeds [17]. Cahoon et al. [22] successfully increased the content of tocotrienols in leaves by overexpressing homogentisic acid geranylgeranyl transferase, the enzyme specifically involves in tocotrienol biosynthesis. While, in this study, we obtained increased levels of α-tocotrienol by introducing an enzyme shared by the tocopherol and tocotrienol biosynthetic pathways, which suggests that *MsTMT* plays a role in redirecting the flux, and thereby, the composition of vitamin E in alfalfa.

The promoter sequence analysis identified a number of light-responsive *cis*-elements present in the *MsTMT* promoter and the subsequent expression analysis showed that *MsTMT* was induced by darkness (Fig. 2D). Darkness also can induce leaf senescence [55] and some studies have demonstrated that vitamin E can affect leaf senescence [50]. Therefore, we tested leaf senescence in the transgenic alfalfa OE16, which accumulated more α-tocopherol and α-tocotrienol than other lines, and found that leaf senescence was delayed in OE16, when compared with wild type. This delay in leaf senescence was determined by the onset of chlorosis dur-

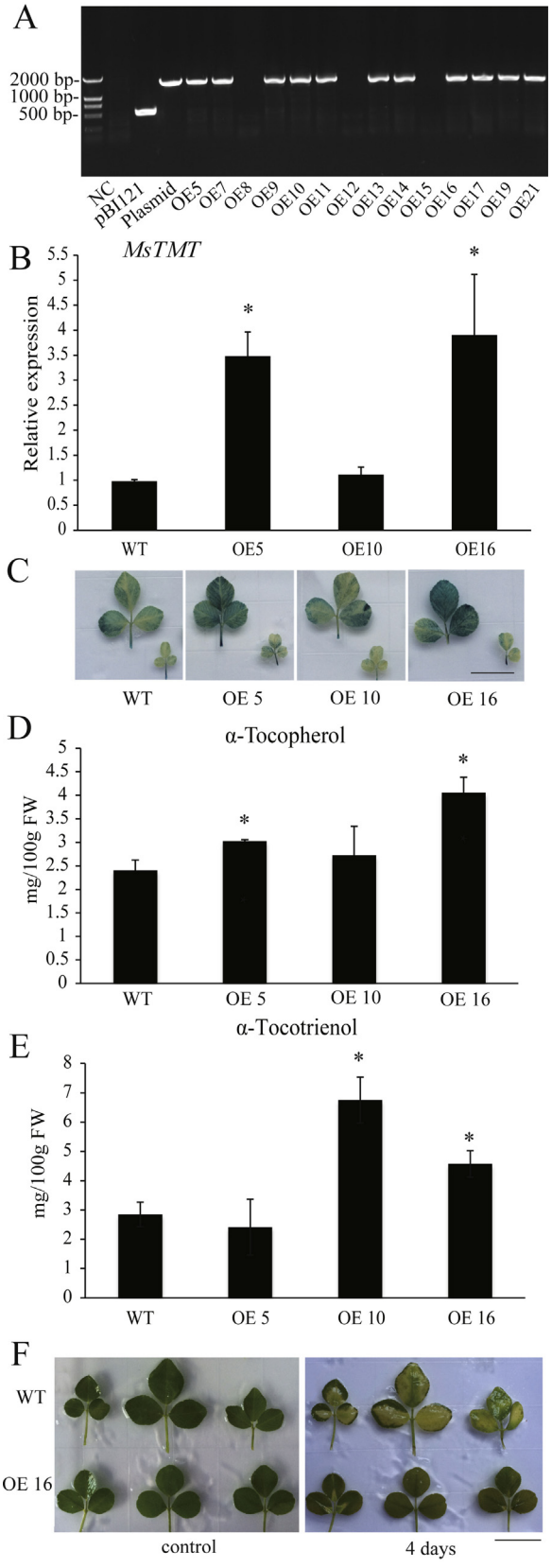


Fig. 7. A. Molecular confirmation of transgenic alfalfa.
DNA was extracted from 2-month-old transgenic alfalfa and used for PCR analysis. NC: Negative control; pBI121: Empty vector; Plasmid: Positive control.
B. Expression analysis of *MsTMT* in alfalfa *MsTMT* overexpression lines (OE 5, OE 10 and OE 16) and Zhongmu No.1 with pBI121 empty vector control plants by qRT-PCR. RNA was extracted from 2-month-old alfalfa. The rest were performed as Fig. 2A.

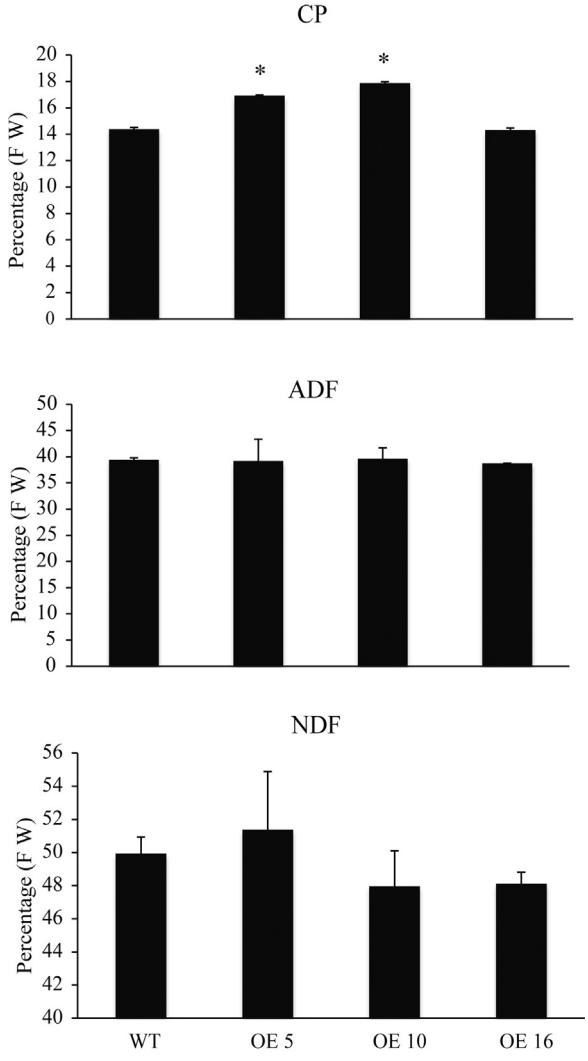


Fig. 8. Levels of Crude Protein (CP), Acid Detergent Fiber (ADF) and Neutral Detergent Fiber (NDF) in *MsTMT* overexpression alfalfa (OE 5, OE 10 and OE 16) and Zhongmu No.1 with pBI121 empty vector. 2-month-old alfalfa leaves were used, data are mean ± SD with three biological replicates.

ing senescence, which occurs as a result of chlorophyll catabolism [56]. Potentially, the increased α-tocopherol or α-tocotrienol level might play a role in protecting chlorophyll from degradation during the leaf senescence process in transgenic alfalfa. Rapid leaf senescence reduces the market value of alfalfa because color is an important post-harvest characteristic for evaluating forage quality. Collectively, these data showed that *MsTMT* overexpression resulted in a delay in leaf senescence, and as such, this approach may aid in extending the stay-green period of alfalfa hay. Another key aspect of forage quality is the actual nutritional value of the hay. Traditional forage quality analyses include Crude Protein (CP), Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF), mineral content, ash and ether extracts [44,57]. In this study, the CP

C. Histochemical GUS staining of 2-month-old Alfalfa *MsTMT* overexpression lines (OE 5, OE 10 and OE 16) and Zhongmu No.1 with pBI121 empty vector. Bar: 1 cm.
D. α-Tocopherol. E. α-Tocotrienol levels in alfalfa *MsTMT* overexpression lines (OE 3, OE 10 and OE 16) and Zhongmu No.1 with pBI121 empty vector. 2-month-old alfalfa leaves were used for the measurement. Data are mean ± SD, each was performed with four biological replicates.
F. Dark- induced alfalfa leaf senescence.
Detached older leaves from 2-month-old *MsTMT* overexpression alfalfa OE16 and Zhongmu No.1 with pBI121 empty vector, surface-sterilized and put in the dark for 4 d. Bar: 1 cm.

content was increased significantly in transgenic lines, indicating that the α-tocopherol and α-tocotrienol content in leaves may be beneficial with regard to the conservation of leaf proteins during hay production. The overexpression of *MsTMT* did not impart any obviously negative impacts on plant yield phenotype, nor were any differences seen in other quality traits, such as ADF or NDF. These results indicate the possibility of quality improvement using transgenic technology in alfalfa and establish *MsTMT* as a candidate gene, which can be used not only in alfalfa, but also, potentially, in other important forage crops.

In conclusion, we cloned a *TMT* gene from alfalfa, and conducted a series of bioinformatical analyses, which implied that this gene was functional. The gene was found to be primarily expressed in leaves and its expression was negatively regulated by light. Overexpression of this gene shifted the majority of γ-tocopherol to α-tocopherol in transgenic *Arabidopsis*, especially in seeds, and transgenic lines were potentially more resistant to osmotic stress. Transgenic alfalfa overexpressing *MsTMT* was also generated, and these lines exhibited increased levels of α-tocopherol and α-tocotrienol in their leaves, as well as having delayed leaf senescence. However, no significant impacts on the nutrient-related traits were identified. Thus, *MsTMT* provides multiple benefits, and can be used as a tool in future projects that are focused on molecular improvements in quality of alfalfa.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.05.004.

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